Title	Automatic fabrication of 3-dimensional tissues using cell sheet manipulator technique
Author(s)	菊地, 鉄太郎
Journal	2015
URL	http://hdl.handle.net/10470/31121

Biomaterials 35 (2014) 2428–2435

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Automatic fabrication of 3-dimensional tissues using cell sheet manipulator technique

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ARTICLE INFO

Article history:
Received 15 November 2013
Accepted 8 December 2013
Available online 23 December 2013

Keywords:
3-D fabrication
Gelatin
Thermally responsive material
Myoblast
Sheet

ABSTRACT

Automated manufacturing is a key for tissue-engineered therapeutic products to become common-place and economical. Here, we developed an automatic cell sheet stacking apparatus to fabricate 3-dimensional tissue-engineered constructs exploiting our cell sheet manipulator technique, where cell sheets harvested from temperature-responsive culture dishes are stacked into a multilayered cell sheet. By optimizing the stacking conditions and cell seeding conditions, the apparatus was eventually capable of reproducibly making five-layer human skeletal muscle myoblast (HSMM) sheets with a thickness of approximately 70–80 μm within 100 min. Histological sections and confocal topographies of the five-layer HSMM sheets revealed a stratified structure with no delamination. In cell counts using trypsinization, the live cell numbers in one-, three- and five-layer HSMM sheets were equivalent to the seeded cell numbers at 1 h after the stacking processes; however, after subsequent 5-day static cultures, the live cell numbers of the five-layered HSMM sheets decreased slightly, while one- and three-layer HSMM sheets maintained their live cell numbers. This suggests that there are thickness limitations in maintaining tissues in a static culture. We concluded that by combining our cell sheet manipulator technique and industrial robot technology we can create a secure, cost-effective manufacturing system able to produce tissue-engineered products from cell sheets.

1. Introduction

Tissue engineering is a technology that can fabricate 3-dimesional (3-D) functional tissues from cells *in vitro*, with the goal of regaining the lost functions of diseased organs. This technology is categorized by cell-based therapy in which its functionality and success is derived from the configurations or structures that can be produced. Until recently, the variety of tissue-engineered therapeutic products has been limited to only a few organs, such as skin or cartilage. In part, this was because the manufacturing of other tissues often requires complicated fabrication methods including very strict aseptic handling that the biological quality is not readily reproducible. Hence, we believe that automated manufacturing facilities will play a key role in tissue-engineered products to make them more common-place and economical.

When constructing 3-D tissues cell sheets are a very useful component. Temperature-responsive culture dishes have surfaces that are covalently grafted with poly (*N*-isopropylacrylamide) and can be used to make cell sheets without applying enzymes [1–5]. In these culture dishes confluently cultured cells detach from the surface as an intact cell sheet by simply reducing the temperature, which causes hydration of the polymer. Since these cell sheets retain their extracellular matrix and the adhesive proteins around them, their structural integrity and mutually adhesive properties allow us to fabricate 3-D constructs by simply stacking them layer upon layer [6–9]. There are many advantages to these multilayered cell sheets for use as therapeutic grafts, which include nonuse of excipients, a stratified structure mimicking physiological tissues, adhesiveness to organs, and efficient local administration of cells. In addition, we think this method is suitable for controlling the thicknesses of cell-dense constructs that might attenuate acute ischemic cell death after implantation caused by insufficient nutrient-waste exchange through solute diffusion. Despite such advantages, the challenges faced to stack cell sheets, either with supportive membranes or by liquid handling, has long hindered

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http://dx.doi.org/10.1016/j.biomaterials.2013.12.014

reproducible fabrication and any precise studies about multilayered cell sheets. However, a recently-developed cell sheet manipulator technique using cell-adherent hydro gels has now made it easier to fabricate 3-D constructs from cell sheets [10], and the method appeared to be enough simple to be done by industrial robots. Hence, we developed an automatic cell sheet stacking apparatus by exploiting the cell sheet manipulator technique, to produce an automatic manufacturing system for tissue-engineered products.

2. Materials and methods

2.1. Temperature-responsive culture dish

Temperature-responsive culture dishes were prepared by the method previously reported [1]. Briefly, a solution of *N*-isopropylacrylamide (NIPAAm) monomer was spread on commercially available tissue culture polystyrene dishes. Then, electron beam irradiation induced the monomer to be polymerized and covalently grafted to the culture surface. These dishes were washed vigorously by cold water to remove any ungrafted monomers, and finally they were sterilized.

2.2. Cell culture

Human skeletal muscle myoblasts (HSMM) were purchased from Lonza (Basel, Switzerland) and propagated through three passages to obtain the necessary cell number. HSMMs were cultured at 37 °C in a humidified atmosphere with 5% CO_2 in SkGM-2 medium (Lonza).

2.3. Cell sheet manipulator

To retrieve the cell sheets from temperature-responsive dishes, plunger-like cell sheet manipulators coated with a cell-adhesive gelatin hydro gel were prepared similarly to our previous report [10], but with several modifications in order to improve reproducibility. To prepare the gelatin solutions, powdered gelatin from porcine skin (Sigma–Aldrich, St. Louis, Missouri, USA) was added to Hanks' balanced salt solution (Sigma–Aldrich) to a final concentration of 6 wt%, neutralized with a small volume of 1 N sodium hydroxide solution (Wako Pure Chemical Industries, Osaka, Japan) and dissolved completely in a 50 °C water bath. Then, the gelatin solution was sterilized by filtration using a bottle top filter with a 0.45 μm pore diameter, and its aliquots were stored at 4 °C until use. On the day before the stacking operation, the aliquot was warmed and melted again in a 37 °C water bath for 30 min, and the gelatin solution was dispensed into 2.5 mL cylindrical polytetrafluorethylene (PTFE) molds. Then, metallic plunger-like manipulators were immediately dipped in the solution and held 3 mm above the bottom. The manipulators were stored at 4 °C overnight to solidify (Fig. 2b). The next day, several minutes before the stacking operation, the manipulators were removed from the molds with the coating of gelatin gel and equilibrated to room temperature.

2.4. Optimization of cell sheet stacking conditions

To shorten the process time, the cell sheet manipulator weights and temperature profiles were manually optimized by measuring the area of cell detachment in each condition, prior to designing the apparatus. HSMMs were seeded in temperature-responsive culture dishes at 1×10^5 cells/cm^2 and cultured overnight. Based on the method previously reported [10], the cells were then stacked in three layers and transferred onto tissue culture polystyrene (TCPS) dishes with manipulators of the various weights (4, 12, 40, and 120 g) and with two types of temperature profiles: with or without a pre-cooling process. In the pre-cooling (−) group, the manipulators were held on the cells throughout the low-temperature treatment at 20 °C and the low-temperature treatment times were varied from 3, 8, 15, 30 and 60 min. In the pre-cooling (+) group, the low-temperature treatment time was fixed at 30 min and the manipulators were placed on the cells at 3, 8 and 15 min before the end of the low-temperature treatment; and the dishes were pre-cooled for 27, 22 and 15 min before applying the manipulators, respectively. After the cell sheets were stacked and transferred to the TCPS dishes, the cells remaining on the temperature-responsive culture dishes were fixed by a 4% formaldehyde aqueous solution for 15 min and stained with 0.08% Crystal Violet Staining Solution (Kanto Chemical, Tokyo, Japan) diluted by 20% ethanol for 15 min at room temperature. After the dishes were washed by distilled water twice, the stained dishes were placed on a white illuminator and pictures were taken by a digital camera. Then, the detachment areas were measured from these pictures using Image J software (public domain, distributed by the National Institute of Health of USA at http://rsbweb.nih.gov/ij/). In addition, the cell sheets transferred on the TCPS dishes were also stained by Crystal Violet to confirm that the transfer was successful.

2.5. Automatic cell sheet stacking apparatus

In pursuit of a method for the stable manufacturing of multilayered cell sheets, an automatic cell sheet stacking apparatus was designed and assembled. The stacking and transfer processes were automated, while the process of washing the melted gelatin was not incorporated because it was not an essential step for this method and required additional machinery for handling liquids. It was designed to accommodate both 3.5 and 6 cm dishes. To preparing for future extensions of this automated system, it was comprised of two modules: an incubation module and a stacking module (Fig. 2a). The incubation module is capable of incubating ten dishes in a humidified 37 °C/5% CO_2 environment. The stacking module handles the dishes and cell sheet manipulators with precise temperature control using Peltier elements. Both modules were connected to each other and installed in a commercially available clean bench (Fig. 2c). The machinery was operated by a laptop PC through a USB interface. All robotic sequences and temperatures were controlled by specially developed software.

2.6. Automatic cell sheet stacking process

The stacking process used by the apparatus was conducted largely as shown in Fig. 1. One modification from the original method previously reported [10] includes the use of seeding rings. Seeding rings were used to prevent cells from adhering at the peripheral areas of the dishes that did not make contact with the manipulator; seeding rings were placed in the dishes and then cells were only seeded within inner area of the ring (4 cm^2). The rings were removed just before stacking which prevents tearing of the cell sheets at the edges of the manipulator. Although the Fig. 1 shows only a four-layer cell sheet being transferred to the last dish, we conducted a number of experiments that varied the destination of the transferred cell sheets as well as the number of layers transferred.

2.7. Optimization of seeding density

To identify the maximum seeding density to reproducibly stack HSMMs, five-layer HSMM sheets were fabricated at various seeding densities by the apparatus and the resulting structures were then examined by a confocal microscopy. HSMMs were seeded in 3.5 cm temperature-responsive culture dishes with seeding rings at 1×10^5, 2.5×10^5, 5×10^5, 1×10^6 and 2×10^6 cells/cm^2 and cultured overnight. On the following day, the cells were marked with live cell fluorescent staining reagents of different colors (CellTracker™) (Life Technologies Corp., Carlsbad, California, USA) according to the instructions provided by the manufacturer. Then, the stained cells were stacked in five layers using the apparatus. The first four HSMM sheets were stacked on the manipulator, and then the four-layer HSMM sheets were transferred onto the fifth dish by melting the gelatin gel. The resultant five-layer HSMM sheets were adhered onto the last dishes and fixed with 4% formaldehyde aqueous solution for 30 min and transparentized by Sca*l*e U2 reagents [11] for 1 week. Cross-sectional views of the multilayered HSMM sheets were subsequently obtained by a confocal laser scanning microscope (LSM 510 META) (Carl Zeiss Microscopy, Cambridge, UK) using the Z-stack method.

2.8. Histological analysis of multilayered HSMM sheets

To examine more precisely the structure of the multilayered HSMM sheets, histological studies were performed for both the detached and adhered samples. To make the detached samples, HSMMs were seeded into 3.5 cm temperature-responsive culture dishes with seeding rings at 1×10^6 cells/cm^2 and cultured overnight. The cells were stacked in five layers and cultured for 1 h on the fifth temperature-responsive dish to allow adhesion. Then, the five-layer construct was again treated with low temperature to detach it from the surface. The detached cell sheets were fixed with a 4% formaldehyde aqueous solution. To make the adhered samples, HSMMs were seeded into 3.5 cm temperature-responsive culture dishes with seeding rings at 1×10^6 cells/cm^2, cultured overnight, stacked in five layers and transferred onto 12 mg/mL fibrin gels (Bolheal®) (Teijin Pharma Ltd., Tokyo, Japan) that were 2 mm thick. The cell sheets transferred onto fibrin gels were fixed with a 4% formaldehyde aqueous solution after 1-h, 2-day and 5-day culture. All samples were embedded in paraffin and subjected to histological examinations, including hematoxylin-eosin (H&E) staining, TUNEL staining and Ki67 immunohistochemistry by established standard procedures.

2.9. Static culture of multilayered HSMM sheets

Lastly, the functionality and survivability of the multilayered HSMM sheets were evaluated by *in vitro* static culture. HSMMs were seeded into temperature-responsive culture dishes with seeding rings at 1×10^6 cells/cm^2 and cultured overnight. The cells were stacked in one, three, and five layers using the apparatus, and then transferred manually onto 10 cm tissue culture polystyrene dishes. The multilayered HSMM sheets were then cultured in 10 mL of SkGM-2 medium. After 1-h culture (referred as day 0), half of the samples were digested by TrypLE Select reagent (Life Technologies Corp.) and then the live cell numbers were counted using Trypan Blue dye and hemocytometers. The other half of the samples was cultured for 5 days. During the culture, the media were changed every 24 h and supernatants were sampled at each medium change. At the end of the culture, the cell sheets were digested and live cell numbers were counted in the same manner as previously described. The collected supernatants were analyzed for glucose, lactate, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), fibroblast growth factor 2 (FGF-2) and stromal cell-derived factor 1α (SDF-1α). The concentrations of glucose and lactate were measured by a BF-6iM medium analyzer (Oji Scientific Instruments, Hyougo, Japan). The concentrations of VEGF, HGF, FGF-2 and SDF-1α

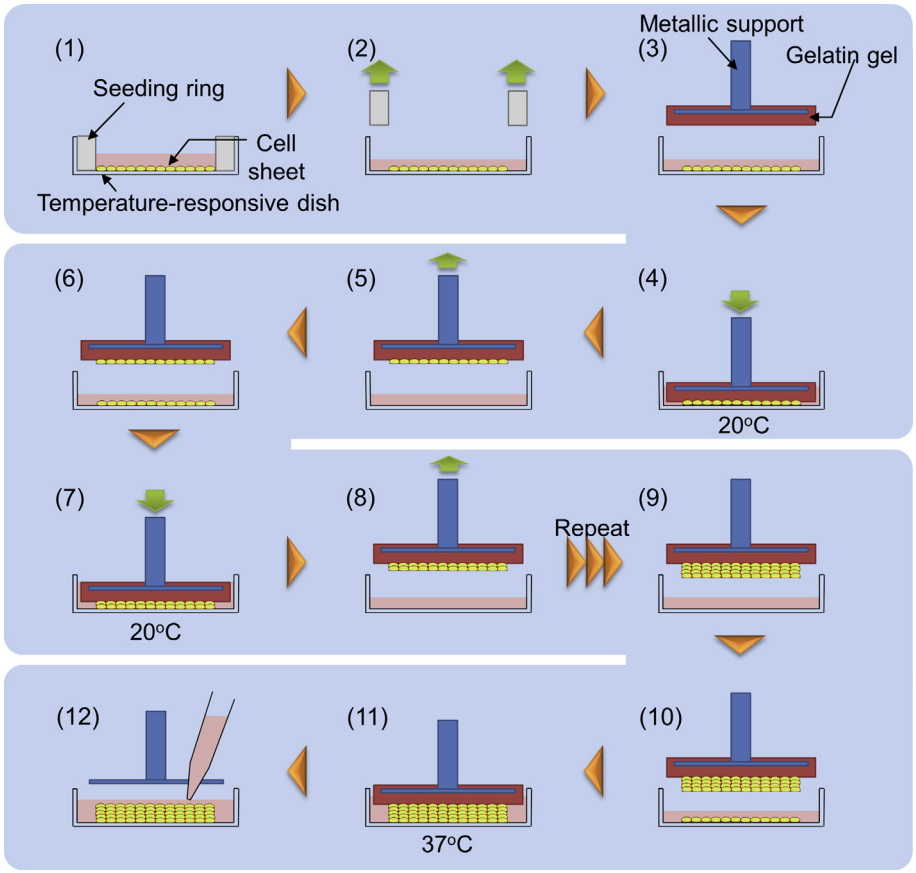

Fig. 1. Automatic cell sheet stacking process. (1–2) Culturing cells on temperature-responsive culture dishes, with seeding rings if necessary, produces intact cell sheets. (3–4) A cell sheet manipulator is placed on the cell sheet and held at 20 °C for a given duration. (5) By picking up the manipulator, the first cell sheet is harvested on the bottom surface of the gel. (6–7) Again, the manipulator is placed on another cell sheet and held at 20 °C. (8) The first and second cell sheets are then stacked on the gel owing to the adhesiveness between the cell sheets. (9) Sequential repetitions of the same procedure for other dishes result in stacking more cell sheets on the gel. (10–11) For the last dish, the manipulator is also placed on last cell sheet, but held at 37 °C instead of the low-temperature treatment at 20 °C. (12) The gelatin gel melts releasing the multilayered cell sheet where it remains on the dish. The melted gelatin will be washed away by warm medium.

were measured using VEGF ELISA Kit (RayBiotech, Norcross, Georgia, USA), IMMUNIS® HGF ELISA Kit (Institute of Immunology Co., Ltd., Tokyo, Japan), Human FGF basic Quantikine® ELISA Kit (R&D Systems, Minneapolis, MinnesotaCity, USA) and Human CXCL12/SDF-1α Quantikine® ELISA Kit (R&D Systems) respectively. To clarify the trends of these measured values with culture time, Jonckheere–Terpstra trend tests were conducted for glucose consumption rate, lactate production rate, VEGF secretion, HGF secretion and SDF-1α secretion. In this test, *p*-values less than 0.05 were considered as significant.

3. Results

3.1. Cell culture and preparation of cell sheets

After the cell expansion through three passages, the ratio of CD56 positive cells was measured as 85% by flow cytometry (data not shown). The inoculated HSMMs successfully attached to the surfaces of the temperature-responsive culture dishes by the following day in all experiments.

3.2. Optimization of cell sheet stacking conditions

The cell detachment areas measured by Crystal Violet staining of the remaining cells on the temperature-responsive dishes are shown in Fig. 3. The detachment area increased with an increase in manipulator weight and stacking time, though the longer stacking time did not compensate for the effect of manipulator weight measured within 60 min. Stacking time refers to the duration for which the manipulators were on the cells. The pre-cooling (+) group yielded larger detachment areas in stacking times of 3 and 8 min, demonstrating that pre-cooling prior to the stacking process promoted detachment of cell sheets. However, the detachment areas in stacking times of 15 min showed no difference between the pre-cooling (+) group and pre-cooling (−) group; implying that a 15-min low-temperature treatment was sufficient to weaken the adherence between the cell sheets and the temperature-responsive surface. Based on these results, we selected the 120 g manipulator and the pre-cooling process to be employed for use in the automatic cell sheet stacking apparatus.

3.3. Automatic cell sheet stacking process

Further adjustments to the apparatus eventually produced the optimum parameters; the stacking times selected were 8 min for the first through third sheets, 10 min for the fourth sheet and 15 min for the fifth sheet, using 120 g manipulators in all cases. The pre-cooling times were fixed at 6 min. The transfer procedure involved an adhering step (5 min at 25 °C) and a melting step (25 min at 37 °C). To construct a five layer cell sheet it took a total of approximately 100 min, which included the initialization of motors, the motion of fetching the dishes and other handling times, in addition to the stacking and transferring times.

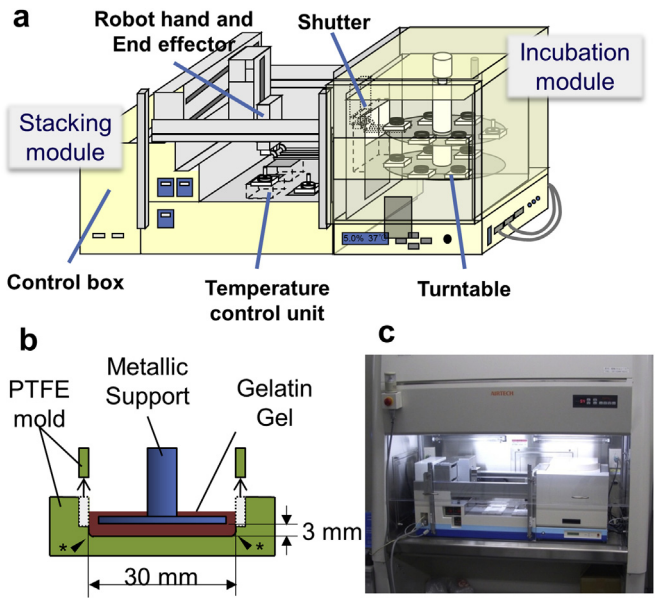

Fig. 2. Devices and apparatuses for cell sheet stacking. (a) Configuration of the automatic cell sheet stacking apparatus. (b) Schematic of the molding gelatin gel. A cylindrical piece of the mold surrounding the gel was removed first in order to achieve the smooth removal of the gel. The edge of the bottom surface was rounded to a radius of 2 mm (arrows with asterisks) to reduce the suction cup effect when detaching from the dishes; hence, the circular area that contacts with the cells was at least 26 mm in diameter (5.3 cm^2). (c) The automatic cell sheet stacking apparatus was installed on a clean bench.

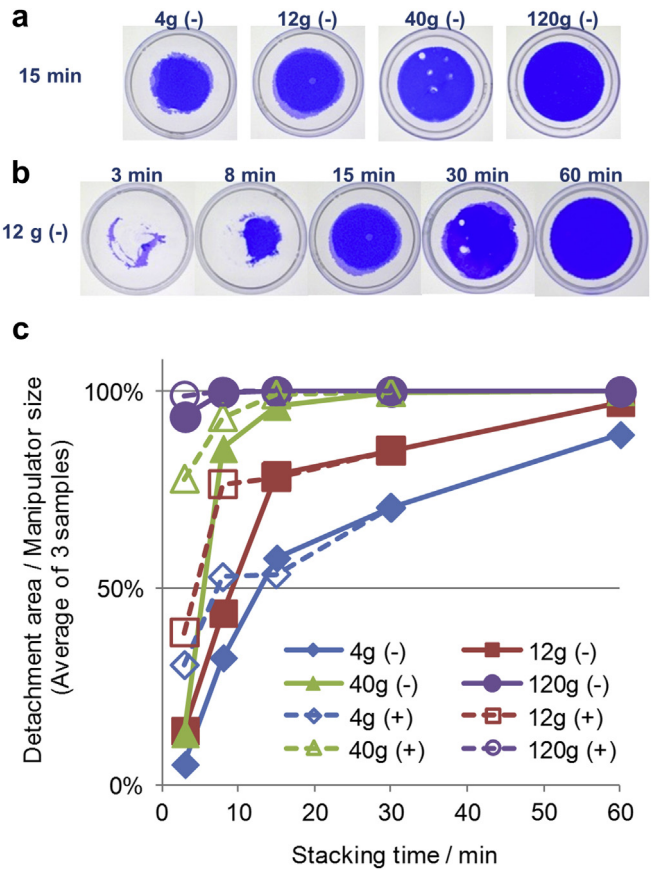

Fig. 3. Measurement of detachment areas. (a–b) Examples of Crystal Violet-stained three-layer HSMM sheets transferred onto tissue-culture polystyrene dishes in the conditional optimization. (a) Cell sheets stacked at 15 min per layer without pre-cooling by each manipulator weight. (b) Cell sheets stacked by 12 g manipulators without pre-cooling for each stacking time. (c) Detachment areas for each stacking condition. The detachment areas were expressed as percentages to the area of the bottom surface of the cell sheet manipulators. Solid lines indicate pre-cooling (−) group. Dotted lines indicate pre-cooling (+) group.

3.4. Optimization of seeding density

The fabrication of five-layer HSMM sheets in seeding densities of 1×10^5, 2.5×10^5, 5×10^5 and 1×10^6 cells/cm^2 were successful, while the fabrication at 2×10^6 cells/cm^2 was not reproducible due to a failure in cell detachment from the fourth and fifth dishes. Cross-sectional views of the five-layer HSMM sheets by confocal microscopy demonstrated their stratified structures (Fig. 4a). The sheet thickness increased with an increase in cell seeding density though the correlation was not proportional, which implies that HSMMs were compressed in higher seeding densities.

3.5. Histological analysis of multilayered HSMM sheets

The histological images of the paraffin sections are shown in Fig. 5. Five-layer HSMM sheets were detached spontaneously from the temperature-responsive surfaces by additional low-temperature treatment followed by gentle rocking of the dishes. The sheets detaching from the dishes gradually shrunk horizontally to nearly half their original diameter (Fig. 4b). The HSMM sheets on fibrin gel all adhered to the gel until the end of the culture. The sections of the HSMM sheets cultured on fibrin gel revealed that in static culture, (i) the cell sheet became slightly thinner, (ii) TUNEL positive cells gradually increased, and (iii) the number of Ki-67 positive non-quiescent cells decreased. In comparison between the detached and adhered sheets, the detached HSMM sheets were thicker than the adhered ones indicating that multilayered HSMM sheets became thicker when there was horizontal shrinkage on detachment.

3.6. Static culture of multilayered HSMM sheets

The live cell numbers at day 5 were almost the same as day 0 in one- and three-layered sheets, while the live cell numbers in five-layered sheets decreased at day 5 by 17.7 ± 2.5% (Fig. 6f). TrypLE Select treatment with gentle pipetting yielded single cell suspensions without aggregations from multilayered HSMM sheets, even after 5-day culture. The live cell numbers just before stacking were almost the same as the seeded cell numbers (data not shown), and the live cell numbers 1 h after stacking (day 0) were equivalent to the sum of the seeded cell numbers in all samples.

In the analysis of culture supernatants, glucose consumption rate and lactate production rate increased from one-layers to three-layers, but the three-layer and five-layer samples showed little difference, and both rates decreased with increased culture time (Fig. 6a, b). The angiogenic factors were studied also and showed that VEGF and SDF-1α secretions were approximately proportional to the number of layers, though HGF secretion was less dependent on the numbers of layers. HGF and SDF-1α secretion had a tendency to increase with increased culture time while VEGF secretion decreased (Fig. 6c–e). The trends with culture time were confirmed by Jonckheere–Terpstra trend test as shown in Table 1. FGF-2 was not detected in any of the samples (<0.1 ng/day).

4. Discussion

The aim of this study was to present evidence of the feasibility of incorporating a cell sheet manipulation technique in the automatic manufacturing of 3-D tissue-engineered therapeutic products.

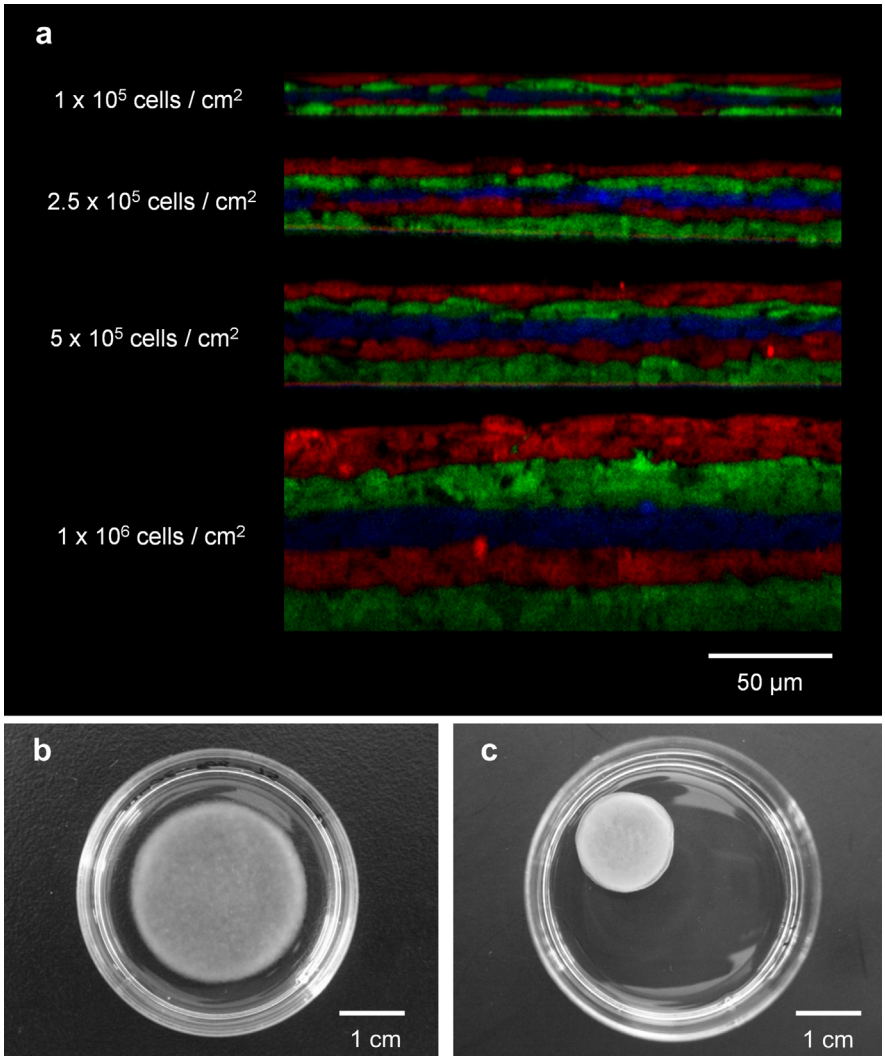

Fig. 4. Macroscopic and confocal observation of five-layer cell sheets. (a) Cross sectional views of five-layer HSMM sheets adhered on dishes as captured by confocal fluorescent microscopy. Each cell sheet has been stained by fluorescent dyes of red for the first and fourth cell sheets, green for the second and fifth cell sheets and blue for the third cell sheet. The images were composed by pseudo colors corresponding to the colors of the dyes. (b) A five-layer HSMM sheet adhered on a dish (1×10^6 cells/cm^2, five layers). (c) A five-layer HSMM sheet detached from a dish (1×10^6 cells/cm^2, five layers).

To accomplish this we developed an automatic cell sheet stacking apparatus to exploit this technique and applied industrial robot technology after we had gained sufficient experience through manual operation and prototypes. We began by stacking the HSMM sheets under various conditions using this apparatus. Conditional optimizations performed manually revealed that heavier manipulators accelerated the retrieval of cell sheets. Since the detachment of cells from the temperature-responsive surface was not likely to be accelerated by the pressure, the result implies that higher pressures promoted adherence between the surface of the manipulator (where the surface presented gelatin gel or a previously stacked cell sheets) and the surface of cells in a dish. Gelatin is a cell adhesive matrix protein and cell sheets harvested from temperature-responsive surfaces retain these adhesive proteins, such as fibronectin [4]. In the cell sheet manipulator technique, the retrieval of a cell sheet from a dish is dependent on maintaining that protein adhesiveness. However, at the start of stacking, a small amount of medium is trapped between the two surfaces by the viscosity of the medium because the two flat surfaces approach in parallel. The trapped medium prevents contact between the two surfaces by supporting the weight of the manipulator. On this supposition, one possible explanation is that higher pressures may force out the medium faster and brings the cell sheets close enough to interact hydrophobically. On the other hand, the 'pre-cooling' process also reduced stacking time simply because this process weakened the adherence between cells and the temperature-responsive surface at the start of stacking. The stacking process succeeds only when the adherence between the manipulator and the cells overcomes the adherence between the cells and the temperature-responsive culture surface. The former depends on pressure, the latter depends on temperature and both depend on process time, if the other conditions are fixed.

Based upon these results, an automatic cell sheet stacking apparatus was designed and assembled. The apparatus eventually became sufficiently compact to be used on commercially available clean benches and it did not require any additional facilities, except for an electric power source and CO_2 supply. These features make it easier to introduce the apparatus into existing cell culture facilities.

In search for the appropriate seeding density required to produce HSMM sheets that were able to be stacked up to five layers, it was found that when we combined higher seeding density and higher layer numbers it would lead to stacking failure. This might

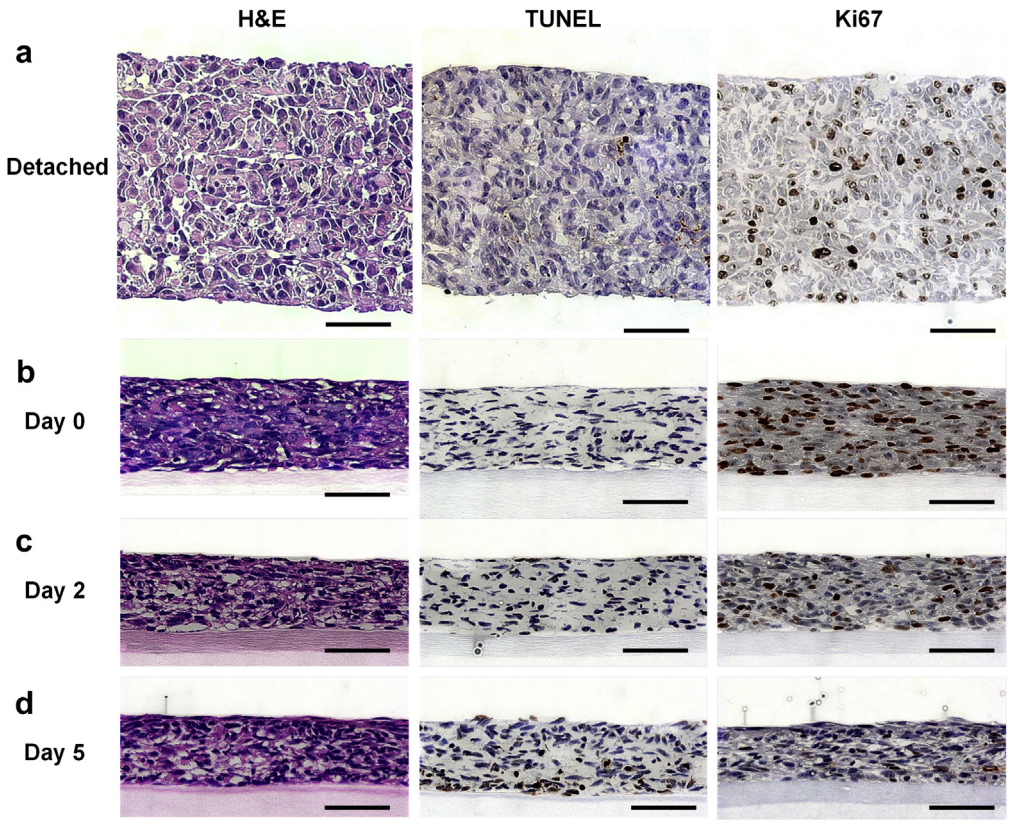

Fig. 5. Histological sections of multilayered cell sheets. (a–d) Cross-sectional views of five-layer HSMM sheets made from 1×10^6 cells/cm^2 cell sheets colored by hematoxylin-eosin staining, TUNEL staining and Ki-67 antibody. The bars in each image correspond to 50 μm. (a) Cell sheets detached from dishes. (b–d) Cell sheets cultured on fibrin gels.

be because the smoothness of the manipulator bottom surface was deteriorated by an increase of cell sheet tensions and nonuniform cell density (it is rather difficult to uniformly seed cells, especially from the center to the periphery in round dishes).

Both topographical analysis by a confocal microscopy and histological analysis of paraffin sections made from five-layered HSMM sheets seeded at the maximum seeding density revealed that their stratified structures did not show any delamination between the layers. We found that this structure was a distinct characteristic of cell sheets stacked by the cell sheet manipulator technique, because multilayered cell sheets stacked by the other techniques invariably used detached and shrunken cell sheets that often included voids or delamination between the layers.

Lastly, the viability and angiogenic potential of multilayered HSMM sheets were confirmed by 5-day static culture. The live cell number after a 1-h culture were equivalent to the seeded cell number used in all the layer variations, while the live cell number after a 5-day culture were equivalent to the seeded cell number in one- and three-layer sheets and slightly lower in five-layer sheets. The glucose consumption rate of the multilayered HSMM sheets was higher in the samples of greater number of layers but the rate per layer was lower in the samples of greater number of layers. Conceivably, these results came from the restricted diffusion of glucose, oxygen, and other nutrients or waste products, even though no apparent tendencies were found in the ratios of lactate production over glucose consumption that would reflect hypoxia. We also investigated the effect on angiogenic factors, and it showed that the greater the number of layers, HGF secretion per layer became lower while VEGF secretion remained almost constant. The contrasting results between VEGF and HGF secretions may also be related to the ischemic situation in multilayered HSMM sheets. It is known that VEGF secretion is induced by hypoxia [12] and HGF secretion is reinforced in normoxic conditions [13]. According to previous reports about the thickness limitations of cell sheets, when neonatal rat cardiomyocyte sheets were implanted subcutaneously, stacking cell sheets over three layers did not grow thicker one month after the transplantation [14]. When human endometrial-derived mesenchymal cell sheets were cultured on a dish, the thickness limitation of layered cell sheets was approximately 40 μm [15]. The five-layer HSMM sheet was approximately 70–80 μm in thickness when observed both in paraffin sections and by confocal microscopy. Although these results cannot be compared directly because cell type and culture conditions were largely different, the phenomenon and the order of thickness limitations were in agreement. One possible solution to solve the thickness limitation is to control the graft thickness, and multilayered cell sheets would be suitable for this purpose. In the cell sheet manipulator technique, the thicknesses of multilayered cell sheets can be controlled by the number of layers and the seeding densities. Another solution is to accelerate blood reperfusion in the grafts after implantation. Mixing endothelial cells into the grafts was effective to promote vascularization [8] and repetitive implantation of thin cell sheets effectively formed thick tissue [14]. The most challenging strategy is *in vitro* vascularization of grafts with an inlet and outlet that can be anastomosed to the host blood vessels. In this strategy, step-by-step cell sheet stacking on a vascular bed was proven to work well [16,17]. On the other hand, glucose consumption rates and lactate production rates decreased during culture in all variations of layer number, implying that the energy consumption gradually decreased by the cells entering quiescence due to high cell densities in the sheets. In another experiment it was demonstrated that glucose consumption of

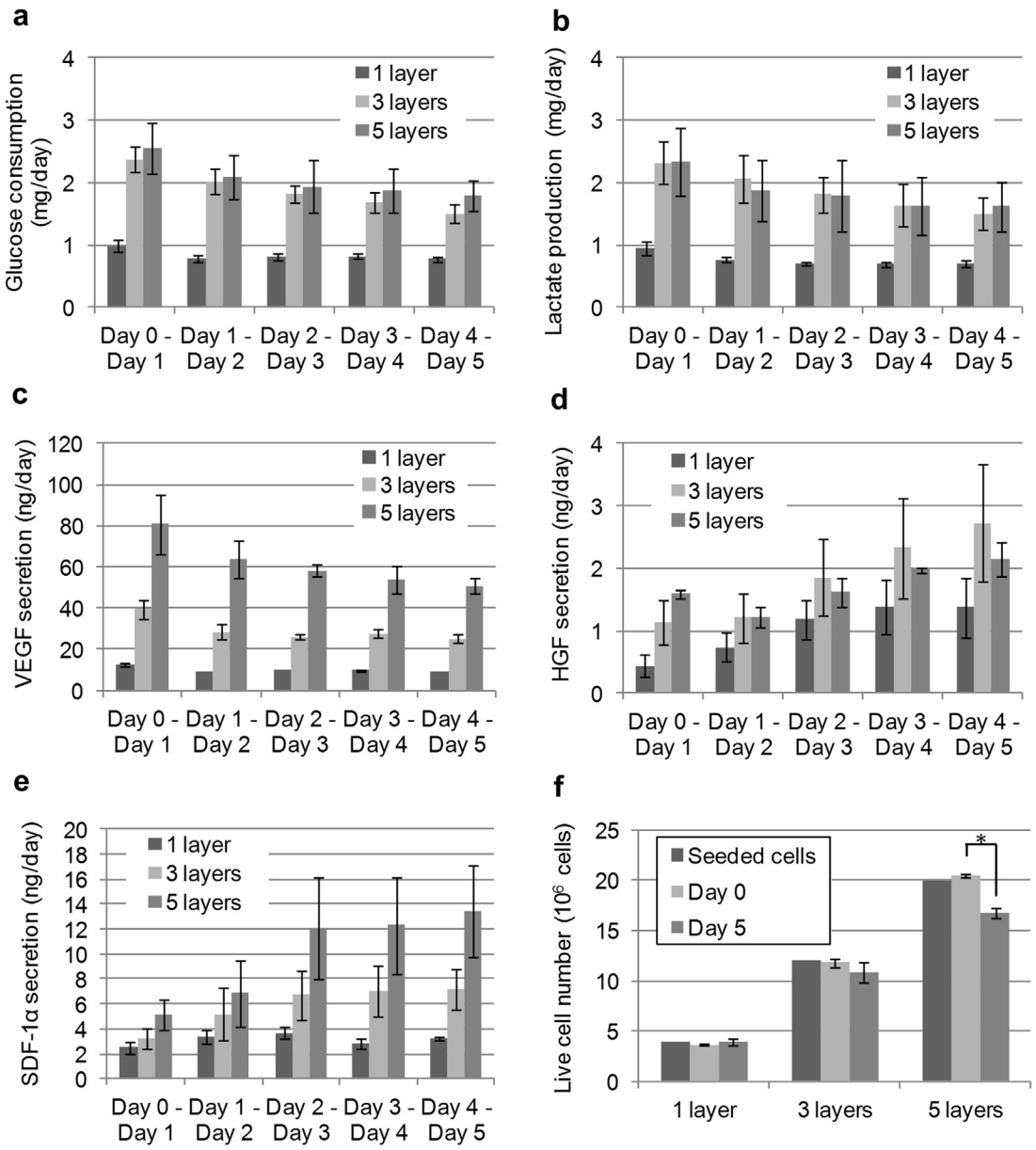

Fig. 6. Static culture of multilayered HSMM sheets. (a–e) Analysis of supernatants at each medium change. (a) Glucose consumption rate. (b) Lactate increase in medium. (c) VEGF secretion rate. (d) HGF secretion rate. (e) SDF-1α secretion rate. (f) Live cell numbers counted in multilayered HSMM sheets. The asterisk means the *p*-value was less than 0.05. Error bars on histograms indicate standard errors of the means.

HSMM sheets in a monolayer decreased along with the seeding densities (data not shown). In parallel, VEGF secretion had a tendency to decrease with culture time which was in contrast to HGF and SDF-1α secretion which increased. These results indicate that multilayered HSMM sheets were subject to some remodeling in static culture. Therefore, it is necessary to develop stable culture conditions for quality control of multilayered cell sheets as a therapeutic product. It might also be possible to improve the quality during a subsequent culture if we incorporate bio-reactors capable of incubating 3-D tissues appropriately.

In this study, our apparatus was tested on HSMM sheets because multilayered HSMM sheets can be an efficient way of implanting myoblasts for treatment of ischemic or dilated cardiomyopathy [18–20]. To decrease the number of cell sheets to implant saves the surgical operation time dramatically; Sawa and co-workers implanted five four-layer myoblast sheets (a total of 20 myoblast

Table 1
Jonckheere–Terpstra trend tests on the static cultures of the five-layer HSMM sheets.

Number of layers	Glucose consumption rate	Lactate increase rate	VEGF secretion	HGF secretion	SDF-1α secretion
One layer	$\searrow p = 0.014$	$\searrow p = 0.00013$	$\searrow p = 0.0011$	$\nearrow p = 0.00038$	N.S.
Three layers	$\searrow p = 0.0000014$	$\searrow p = 0.0017$	$\searrow p = 0.0017$	$\nearrow p = 0.00038$	$\nearrow p = 0.0042$
Five layers	$\searrow p = 0.020$	$\searrow p = 0.014$	$\searrow p = 0.0017$	$\nearrow p = 0.0042$	$\nearrow p = 0.00064$

$\searrow$: Decreased with culture time, $\nearrow$: increased with culture time, N.S.: no significant trend, p: critical rates.

sheets) in their report. On the other hand, our apparatus may be also applicable to cell sheets made from other cell types though they require different optimization of process conditions and thicknesses of multilayered cell sheets. In addition, the cell sheet manipulator technique may be useful for tissue models comprised of several types of cell sheets just as many normal tissues often show in native physiological situations, such as combinations of epithelial cells and stromal cells.

In the past decade, tissue-engineered therapeutic products made from cells and scaffolds have been widely investigated, and several products become commercially available. In this situation, the goal of automation in tissue-engineering is to pursue better efficiency of production and to diminish the risk of failures. Manufacturing of tissue-engineered grafts has been largely dependent on aseptic liquid handling techniques on clean bench established in the last century. Industrial robots and facilities may provide robustness and reproducibility by decreasing the risk of human errors and contaminations due to human interventions in biological processes [21]. Meanwhile, a hurdle remains in familiarizing researchers about automated fabrication: Single process apparatuses should be installed in conventional clean rooms because the preceding and subsequent processes still have to be done by hand. Although our apparatus was only able to succeed in the cell sheet stacking process, the other processes including cell-isolation and cell-expansion have been also investigated separately in our research group. If all these apparatuses could be integrated, an aseptic facility would be simpler than conventional facilities by removing the need for aseptic operations in separate clean benches. This kind of integrated, automated design might be called a fully-automated manufacturing system of tissue-engineered products.

5. Conclusions

Our newly developed automatic cell sheet stacking apparatus was able to fabricate multilayered human skeletal muscle myoblast sheets stably. This presents the possibility that by combining our cell sheet manipulator technique and industrial robot technology we can create a secure and cost-effective manufacturing system able to produce tissue-engineered products from cell sheets.

Acknowledgments

We would like to thank all who have supported this work. We are especially grateful to the colleagues in CellSeed for much advice on designing the apparatuses and devices. This study was supported in part by the New Energy and Industrial Technology Development Organization (NEDO) under the Ministry of Economy Trade and Industry of Japan, and in part by the Cabinet Office, Government of Japan and the Japan Society for the Promotion of Science (JSPS) through the Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program). We thank Allan Nisbet for his useful comments and editing assistance.

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